Microreview

Protein export from *Plasmodium* parasites

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Summary

Many prokaryotic and eukaryotic intracellular pathogens survive by altering the host cell through the export of proteins. In contrast to the well-studied prokaryotic export systems, knowledge of protein export in eukaryotic pathogens is scant. The recent discovery that a short protein sequence targets a protein for export from the malaria parasite *Plasmodium falciparum* has shed light on the possible mechanism of proteins export and has allowed the preliminary identification of several hundred exported proteins. Among the exported proteins are the members of the paralogous protein families, previously identified exported proteins and many uncharacterized proteins. The interaction of the parasite with the host cell is thus much more complex, and involves more parasite proteins, than previously thought.

Intracellular pathogens enter and survive within their host cell by modifying it through the export of effector proteins. Examples of pathogen-induced changes are alterations in membrane transport to avoid fusion with and degradation in the lysosomes (Hernandez *et al.*, 2004) or to acquire necessary nutrients (van Ooij *et al.*, 2000), prevention of apoptosis in the host cell (Abramovitch *et al.*, 2003) and downregulation of signalling in the host cell (Espinosa and Alfano, 2004). Failure to export proteins blocks the pathogen from replicating. Pathogens that reside in a membrane-bound compartment within the host cell face the problem that the effectors need to pass not only their own membrane but also the membrane of the vacuole. Several protein transport systems dedicated to this task have evolved, the best studied of which are the Type III and Type IV secretion systems of bacteria, and the identity and function of many of the effectors are known (for review, see Cambronne and Roym, 2006).

Much less is known about secretion of proteins from intracellular eukaryotic pathogens. *Plasmodium falciparum*, a protozoan parasite, causes multiple changes in the host red blood cell, including an increase in adhesiveness to the endothelium and its membrane permeability and a decrease in its deformability. The parasite proteins responsible, and the mechanism of export are, with some exceptions, not known. The recent discovery of a host targeting (HT) signal (also referred to as *Plasmodium* export element-PEXEL) (Hiller *et al.*, 2004; Marti *et al.*, 2004), a short sequence in proteins that promotes their export from the parasite into the host cell, is the beginning of the elucidation of the protein export pathway in *Plasmodium* species, and has allowed provisional identification of proteins exported by the parasite into the host cell (the ‘secretome’) (Hiller *et al.*, 2004; Marti *et al.*, 2004). This review will discuss the process of protein export in *Plasmodium* species, including the role of the HT, the members of the host-targeted secretome, potential roles of these members in the proliferation and pathogenesis of the parasite, and discuss the possible mechanisms for the export of the effectors and the evolutionary conservation of this eukaryotic pathogenic protein translocation system.

*Plasmodium*, malaria and protein export

*Plasmodium falciparum* is one of the causative agents of malaria. This disease is more widespread than ever, afflicting nearly half a billion people yearly, leading to one and a half million deaths (Breman, 2001). The symptoms of malaria depend greatly on the genetics of the parasite and the host, but generally consist of chills and fever, and can include anaemia, hypoglycaemia, coma and even death. In each case they are a consequence of the infection of the host erythrocytes (red blood cells) by the parasite. The parasite invades the erythrocytes using an elaborate myosin-based motility complex (for review, see Sibley, 2004), which is required as the erythrocyte is devoid of any endocytic machinery. Subsequently the parasite resides in a membrane-bound compartment, the parasitophorous vacuole (PV), where it undergoes several rounds of nuclear division (see Fig. 1 A and B). Forty-eight hours post infection, the parasite is divided into 16–32 progeny, which are released upon rupture of both the PV and the host erythrocyte (Fig. 1B).
During the course of the intraerythrocytic cycle, several membrane-bound compartments are formed inside the erythrocyte. The Maurer’s Clefts (see Fig. 1) are found underneath the surface of the erythrocyte and contain many parasite-derived proteins, including those destined for the surface of the erythrocyte, such as PfEMP1 described below). Also, a tubovesicular network (TVN) is formed within the erythrocyte, which may play a role in the uptake of nutrients by the parasite (Lauer et al., 1997).

The parasite also induces multiple changes in the properties of the host cell membrane, of which the parasite-induced adhesiveness to the endothelium is the best described (Udeinya et al., 1981). This property, which is thought to underlie many of the symptoms of malaria, results from the appearance of parasite-derived ‘knobs’ on the surface of the erythrocyte (Aikawa et al., 1983; Crabb et al., 1997); it leads to sequestration of infected erythrocytes in the peripheral vascular system and so prevents clearance in the spleen. Knobs are large proteinaceous structures consisting of the parasite-derived erythrocyte membrane protein (PIEMP1) (Baruch et al., 1995) and knob-associated histidine rich proteins (KAHRP) (Pologe and Ravetch, 1986). PIEMP1 is encoded by the large var family; each var gene product differs in its antigenic properties (Joergensen et al., 2006). Thus by changing the var allele that is expressed, the parasite can alter the antigenic properties of the infected cell, and so avoid clearance by the host immune system. The parasite encodes several other families of paralogous proteins of unknown function, the RIFINs (Cheng et al., 1998), STEVORs (Cheng et al., 1998) and MC-2TM (Sam-Yellowe et al., 2004). These proteins consist of conserved N- and C-terminal regions, separated by two transmembrane domains that flank a hypervariable region. By switching the alleles of RIFIN,
STEVOR, MC-2TM and PIEMP1 that are transcribed, the parasite can evade immune surveillance, as the variable region of each member of the family has different antigenic properties. This implies that the variable regions of these proteins are exposed to the immune system. For this to occur, they have to be exported from the parasite and exposed at the erythrocyte plasma membrane surface. This has been unequivocally established only for PIEMP1. Studies on RIFINs also suggest that they are exported exposed at the erythrocyte plasma membrane surface. This has been recently questioned by Khattab and Klinkert (2006).

Further exposure at the erythrocyte surface could likely be true for STEVOR and MC-2TM proteins as well, but presently there is no robust evidence demonstrating this.

Prior to translocation to the erythrocyte membrane, the *Plasmodium* proteins bound for export must traverse two membranes, the plasma membrane of the parasite and the PV membrane (PVM; see arrow 2 in Fig. 1A). Until recently only few proteins, both soluble and membrane-bound, were shown to be exported and the mechanism by which it occurred was unknown. The discovery of the HT signal has shed light on the possible mechanism of export and allowed the identification of additional exported proteins.

**Requirements for protein export in *Plasmodium***

Simplistically, the export of a protein into the host cell can be divided into two steps: secretion of the protein into the PV and the transport of the protein past the PVM (see Fig. 1A). Two lines of evidence suggest that the first step occurs through the endoplasmic reticulum (ER)-based secretory pathway. First, many, but not all, exported proteins carry a clearly recognizable ER-type signal sequence (Lingelbach, 1993), the hydrophobic sequence at the N-terminus of the secreted proteins that directs proteins to the transport apparatus in the ER. In an experimental system investigating the export of histidine-rich protein II (HRPII) fusions to green fluorescent protein (GFP), it was shown that the signal sequence of HRPII could direct the secretion of GFP to the PV (Lopez-Estrano et al., 2003). By immunoblotting, unprocessed precursor could be detected along with the processed mature protein, demonstrating that the fusion was transported through the ER (Lopez-Estrano et al., 2003). Conversely, an HRPII-myc fusion that lacked a signal sequence was not secreted, but detected in the parasite cytosol, indicating that a functional signal sequence is required for export (C. Lopez-Estrano and K. Haldar, unpublished). Several exported proteins, such as glycoporphin-binding protein-130, KAHRP, and ring-expressed surface antigen (RESA), contain an unusual recessed signal sequence, located more than 50 amino acids from the N-terminus (reviewed in Nacer et al., 2001). Why some proteins have a recessed signal sequence is unclear; replacement of a recessed signal sequence with a standard signal sequence did not lead to alteration of export (Lopez-Estrano et al., 2003). The major virulence factor PIEMP1 is exported without an obvious signal sequence at the N-terminus. Its export has been explained by the presence of an internal start-transfer sequence (this is a sequence internal in the protein that can direct the transfer into the ER), and fusions of short regions of PIEMP1 containing the transmembrane domain to GFP are indeed exported (Hiller et al., 2004; Marti et al., 2005). Additional evidence for the use of the ER-based secretory apparatus is the Brefeldin A (BFA) sensitivity of protein export. BFA is a fungal metabolite that prevents anterograde transport in the secretory pathway, effectively blocking secretion of proteins. In BFA-treated parasites, secreted proteins are found trapped in the parasite (Cray and Haldar, 1992), in several cases colocalizing with ER markers (Wickham et al., 2001). There have been reports of a second ‘alternative’ secretion pathway (Benting et al., 1994; Wiser et al., 1997) in which the secretion of exported protein involves a separate, BFA-sensitive secretory organelle, but its exact nature remains unclear (Mattei et al., 1999).

The second step of export, past the PVM, has long been mysterious. The recent discovery of the HT signal, a sequence necessary for the export of proteins into the host cell, was a great step forward in the elucidation of the mechanism of export and identification of secreted proteins. It was previously known that the N-terminal 64 amino acids (including the signal sequence) of HRPII were sufficient to target GFP to the host cell [this sequence is referred to as the vacuolar targeting sequence (VTS), see Fig. 1D, left] (Lopez-Estrano et al., 2003). Subsequently, Hiller et al. (2004) and Marti et al. (2004) used different approaches to narrow down the sequence required for export and showed it contained a highly conserved core of only five residues. Hiller et al. (2004) used the software package MEME to find conserved residues in the VTS of five soluble exported proteins. This identified an 11-amino-acid sequence that consists of a five-amino-acid core, surrounded by less conserved, but still restricted, sequence. Marti et al. (2004) aligned the signal sequences in the N-termini of 10 exported proteins, including the integral membrane proteins RIFIN and STEVOR, and noticed the distinct conservation of three residues in a stretch of five amino acids. Despite the different approaches, both groups identified the core sequence RxLxE as required for export; replacement of the R, L or E residue with A abolishes transport (Hiller et al., 2004; Marti et al., 2004). The requirement for specific amino acids at the x-positions is less stringent, although not completely random. At the second position is often a hydrophobic residue, such as I or L, while position
4 also contains sequence information, albeit less stringent than at positions 1, 3 and 5. It should be pointed out that while the two groups identified the same sequence, the HT signal and PEXEL differ in their exact definition. The former is a matrix-based motif that assigns a probability to each position to predict export whereas the PEXEL is a linear motif. A direct comparison of the HT signal and PEXEL found that they have similar success in predicting the export of proteins (Sargeant et al., 2006).

The HT predicts that the residues surrounding the five-amino-acid core are also important for export, although only a low level of sequence conservation was detected. Experimental evidence with protein fusions in which GFP immediately followed the five-amino-acid core showed this to be correct. Different fusion proteins were not exported, but insertion of a linker restored the ability of the HT to direct export of the fusion protein to the host cell (Lopez-Estrano et al., 2003; Knuepfer et al., 2005a). Either the sequence following the five-amino-acid core encodes more cryptic sequence information that was not recognized in the initial alignments of the proteins, or there is a structural requirement (e.g. absence of secondary structure) that has no obvious sequence conservation. Mutational analysis of the residues N-terminal to the five-amino-acid core also revealed a sequence requirement (Bhattacharjee et al., 2006).

The location of the HT signal within exported proteins is highly conserved; in all experimentally verified systems it is located within the N-terminal 100 amino acids following the signal sequence (the exception being PIEMP1, discussed below). Analysis of the distance between the putative end of the signal sequence in the secretome and the HT showed a spread ranging from 15 to about 32 residues (Fig. 1E).

The mechanism by which the HT signal promotes translocation of proteins into the host cytosol is still unclear. The discovery of the HT signal in both soluble and integral membrane proteins makes its role more difficult to conceptualize, as pointed out by Lingelbach and Przyborski (2006). They propose that the HT signal functions as early as the ER, but does not rely on entry into the ER. Instead, in their model the HT signal mediates interaction with the outer leaflet of the ER membrane, which allows it to be carried along the exocytic pathway to the parasite plasma membrane, where it is packaged in transport vesicles that transfer the protein through the PV space. Fusion of these vesicles with the PVM would then release the protein into host cell cytosol. While attractive because it addresses the need for the HT signal in both soluble and integral membrane proteins, it does not explain why so many of the exported proteins have a functional signal sequence (which in the case of HRPII is required for export), and what the mechanism is for release of the protein from the membrane once exposed to the host cell cytosol. As described below, an intriguing model for export of PIEMP1 has been proposed, but its applicability to other transmembrane proteins is not clear.

**HT signal/PEXEL in PIEMP1**

Hiller et al. (2004) and Marti et al. (2004) differ in their identification of the sequence in PIEMP1 required for export. Marti et al. (2004) placed the PEXEL within the N-terminal 35 amino acids, while Hiller et al. (2004) identified an HT signal about 300 amino acids from the N-terminus. Both groups validated the identified sequence with GFP fusions, which were exported to the host cell, and point mutations, which were not. The HT signal identified by Hiller et al. resides within the Duffy-binding ligand (DBL) domain of PIEMP1. Recent studies on the structure of PI-EBA-175 (which contains a DBL domain) predict that the HT signal would not be exposed on the surface of the DBL domain. However, recent work also suggests that PIEMP1 is not in its final mature conformation while in transit to the erythrocyte membrane; rather it is projected to be associated with chaperones across the PV and erythrocyte cytoplasm (Knuepfer et al., 2005b; Papakrivos et al., 2005). Thus, it is possible that PIEMP1 contains several regions that are recognized as HT signal. In contrast to other exported proteins, the HT signal is separated from the sequence used for ER translocation by several hundred amino acids which in turn is located C-terminal to the HT signal.

Lingelbach and colleagues showed by elegant biochemical experiments that PIEMP1, despite the presence of a transmembrane sequence, is found in a soluble form, as well as an integral membrane form within the parasite (Papakrivos et al., 2005). Treatment with BFA increased the soluble fraction of PIEMP1, indicating that membrane insertion of PIEMP1 takes place after the protein is transported out of the ER. In addition, a fusion of the PIEMP1 transmembrane domain with a STEVOR fused to an ER-retention signal was found to be an integral membrane protein (Przyborski et al., 2005), perhaps indicating that PIEMP1 is a unique case, rather than the norm. Papakrivos et al. (2005) attributed the solubility of PIEMP1 to its unusual transmembrane domain, which is not recognized as such by several prediction programs; this in contrast to the transmembrane regions of STEVOR, which are recognized as such. Alternatively, the
presence of the ER retention signal may influence processing of the STEVOR protein.

Identification of exported proteins

Using the HT signal/PEXEL as a marker for exported proteins, Hiller et al. and Marti et al. provisionally identified the *P. falciparum* proteins putatively exported via the export motif. The resulting lists of exported protein do not overlap completely, but both groups identify surprisingly large sets of proteins (referred to as the 'exportome' or 'secretome'). The difference between the lists can be attributed to several factors, such as differences in the definition of the PEXEL and the HT signal, and predictions attributed to several factors, such as differences in the cytoskeleton or are part of the knob complex. In addition, entry into the host cell, which does not contain any membrane transport machinery, requires the removal of the host cytoskeleton at the entry site. Several interactions of parasite proteins with the host cytoskeleton have been demonstrated: mature parasite-infected erythrocyte surface antigen (MESA) binds to band 4.1 (Lustigman et al., 1991), KAHRP to ankyrin (Magowan et al., 2000), spectrin (Pei et al., 2005) and actin (Oh et al., 2000) and there is evidence that HRPII can bind actin (Benedetti et al., 2003). Studies comparing the heat tolerance of uninfected erythrocytes and erythrocytes infected with control or resa-deleted parasites showed that RESA protected the erythrocytes from a temperature-induced decrease in size (Silva et al., 2005). As fever is a common symptom of malaria, this cytoskeletal stabilization is likely to play an important role in the survival of the parasite. The functions of other interactions are not known, but they are also likely to stabilize the erythrocyte during the expansion of the parasite.

KAHRP and PfEMP1 form the structural components of the knobs; the role for PfEMP3 remains unclear (Waterkeyn et al., 2000). The extracellular portion of PfEMP1 contains several domains that bind host ligands, leading to adherence of the infected erythrocyte to the endothelium. Several regions of KAHRP have been shown to bind to the cytoplasmic region of PfEMP1 (Pei et al., 2005), and the two proteins colocalize in both Maurer’s Clefts and knobs (Wickham et al., 2001; Rug et al., 2006). Deletion of the gene-encoding KAHRP causes the infected erythrocyte to become knob-less (Crabb et al.,

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### Table 1. In silico annotation of Plasmodium proteins containing a signal sequence and RxLxE/D/Q HT signal

<table>
<thead>
<tr>
<th>In silico annotation of Plasmodium proteins containing a signal sequence and RxLxE/D/Q HT signal</th>
<th>No. of proteins&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIFIN (paralogous family)</td>
<td>165</td>
</tr>
<tr>
<td>STEVOR (paralogous family)</td>
<td>32–34</td>
</tr>
<tr>
<td>Kinases (R45-FIKK kinases)</td>
<td>18</td>
</tr>
<tr>
<td>DNAj/heat shock (Hsp40, 2; RESA and RESA-related, 8; other, 7)</td>
<td>17</td>
</tr>
<tr>
<td>MC-2TM (paralogous family)</td>
<td>13</td>
</tr>
<tr>
<td>Phosphatases</td>
<td>3</td>
</tr>
<tr>
<td>ABC transporter</td>
<td>1</td>
</tr>
<tr>
<td>Knob-associated histidine-rich protein (KAHRP)</td>
<td>1</td>
</tr>
<tr>
<td>Other proteins known to be exported to the erythrocyte (GBP130, 3; PIEMP2 MESA, 1; PIHRP1, 1; PIEMP3, 1)</td>
<td>6</td>
</tr>
<tr>
<td>PFEMP1 cell surface adhesin (antigenic family, no leader signal sequence, internal signal sequence)</td>
<td>59</td>
</tr>
<tr>
<td>Hypothetical unknown (includes some antigenic families with 10 or fewer members)</td>
<td>–80–190</td>
</tr>
</tbody>
</table>

<sup>a</sup> Assignments based on Hiller et al. (2004); Marti et al. (2004); Sam-Yellowe et al. (2004); Ward et al. (2004); Schneider and Mercereau-Puijalon (2005); Sargeant et al. (2006) and http://fozzie.pathology.northwestern.edu/cgi-bin/PlasmoHT/index.cgi.

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As mentioned above, KAHRP also binds cytoskeletal proteins, which likely plays a role in anchoring the knobs to the host cytoskeleton.

In addition, a family of 18–20 kinases (named R45-FIKK) contains the HT signal (Schneider and Mercereau-Pujol, 2005), of which the best-characterized member is R45 (Bonnefoy et al., 1992). No biochemical evidence of kinase activity has been demonstrated, but the active sites have been well conserved. Neither target nor regulatory input has been reported, but the identification of parasite-encoded signalling proteins indicates that the parasite could actively monitor the conditions within the host cell. Alternatively, the kinases may be modulating the host cell by activating or inactivating host cell components, for example, in the cytoskeleton. It is interesting that many of the kinases are detected in the merozoite (the free, infectious) stage, indicating a role in the early remodelling of the host cell. Hiller et al. also reported the export of a phosphatase, again possibly to monitor the physiological state within the host cell or modulate host cell components.

Many exported proteins contain a DnaJ domain (J-domain), a signature domain of heat shock proteins (Hsps). Hsp40 and its homologues contain a J-domain and function as a cofactor for Hsp70. Sargeant et al. identified 19 different J-domain proteins that are putatively exported, while Hiller et al. identified 17, of which the export of RESA and PFE0055c has been experimentally verified (Hiller et al., 2004; Haldar et al., 2006; Sargeant et al., 2006). A possible role for the Hsps in export will be described in more detail below.

The HT-dependent secretome is almost as informative for the proteins not included. It contains no proteins involved in membrane traffic, even though several [PI3Sar1p (Albano et al., 1999), PI4Sec31p (Adisa et al., 2001) and PI4Sec23p (Wickert et al., 2003) and a COPII pathway (Adisa et al., 2002; Taraschi et al., 2003)] are reported to be detected in the host cell. Also, no SNAP and SNARE homologues, proteins involved in the fusion of membranes, contain the HTS. There is evidence that there are parasite-derived transport vesicles inside the erythrocyte, but it is not clear what regulates their fusion. Several known exported proteins, such as the Maurer’s Cleft-resident proteins skeleton-binding protein 1 and Maurer’s Cleft-associated histidine-rich protein, do not contain an HT signal. Possibly these proteins are exported in complex with HT signal-containing proteins, or by an HT signal-independent pathway.

Many of the exported proteins are synthesized both at very late and at very early stages of the intraerythrocytic infection (Marti et al., 2004). Such an expression pattern is often seen for proteins used early in infection, and presumably these proteins are exported soon after parasite entry. This makes sense in light of the fact that this is also the time the parasite has to establish itself within the erythrocyte and requires the most remodelling. However, there are some notable exceptions, such as MESA, which is expressed maximally between 18 and 30 h after invasion, STEVOR (peak expression 20–24 h after invasion) and KAHRP (10–20 h after invasion). Many experimental systems have used the Calmodulin promoter (Lopez-Estrano et al., 2003; Hiller et al., 2004), which is maximally transcribed late during the trophozoite stage, and export has been detected in these systems. Hence, the export system is active soon after entry of the parasite into the host cell and remains active until the late intraerythrocytic stages.

While most of the identified exported proteins are synthesized during the intraerythrocyte stage of infection, several are made during the sporozoite stage (the initial stage of human infection, which takes place in the liver) and in the gametocyte stage (the first stage of the sexual cycle, also in erythrocytes). Presence of the HT signal in the sporozoite proteins circumsporozoite protein (CSP) and liver stage antigen 3 (LSA-3) provides an indication that the parasite also actively remodels the host hepatocyte (a nucleated cell), although there are no published reports yet showing that either protein is exported. Both these proteins are malaria vaccine candidates, with CSP in advance testing (Enosse et al., 2006). CSP does not have a two-exon structure (that is manifest by many HT signal containing proteins). In this regard it is similar to validated HT signal-containing proteins such as Hsp40 (four/five exons) and PFE1615c (one exon) as well as the R45-FIKK kinases (three exons), a family of 18–20 proteins that represent a major P. falciparum-specific expansion of HT signal containing proteins. Williamson and colleagues showed that the gametocyte-specific protein Pfg14.744 is exported (Eksi et al., 2005), and there is evidence that the Pf 11-1 gene product is also exported during the gametocyte stage (Scherf et al., 1992). The export of proteins and HT signal is thus likely to be important in all stages of human infection.

The expression so far has been on proteins exported through the constitutive (ER- and Golgi-based) secretory pathway. Plasmodia contain several other regulated secretory organelles, the rhoptries, micronemes and dense granules, referred to as the apical organelles (see Fig. 1B, i). These are required in the invasion process and are subsequently lost. RESA is secreted through the dense granules (Aikawa et al., 1990; Culvenor et al., 1991), opening up the possibility that additional proteins in apical organelles are exported. Interestingly, the HT signal in RESA varies slightly from that described earlier (RxLxGE) (Marti et al., 2005), and expression of RESA at a time when no dense granules are present leads to accumulation of RESA in the PV, without further export (Rug et al., 2004). Export of organellar proteins has impli-
cations both for the timing of the appearance of the export pathway and the mechanism of export.

Export and secretome in other Plasmodium and non-Plasmodium species

Protein export has been studied primarily in *P. falciparum*, but other species of *Plasmodium* also export proteins. Examples include members of the conserved pir family, which includes the *Plasmodium yoelii* yr, *P. berghei* bir and *P. vivax* vir family (Janssen et al., 2004). Conservation of HT signal-dependent export was shown by heterologous expression of *Plasmodium gallinaceum* and *P. vivax* protein in *P. falciparum* (Marti et al., 2004) and a *P. falciparum* HT signal-GFP fusion in *P. berghei* (C. van Ooij, unpublished). With this knowledge, secretomes have been predicted for the *Plasmodium* species for which genome sequence information is available (Sargeant et al., 2006). Interestingly, the *non-falciparum* species tend to have a smaller predicted secretome, indicating a radiation of exported proteins in *P. falciparum* (Sargeant et al., 2006). A subset of exported proteins is conserved in all sequenced species. These proteins likely perform a core set of functions required for every species in the remodelling of the host erythrocyte. A list of these proteins was published (Sargeant et al., 2006) and surprisingly, none of these proteins are annotated, despite their expected essential function for the parasite. Another subset of exported proteins [such as the pHIST family (Sargeant et al., 2006)] is conserved in species that share the same host. The function of these proteins is also not known, but they are likely involved in host-specific adaptation. Finally, many exported proteins are unique to one species of *Plasmodium* and likely responsible for the species-specific symptoms.

Bioinformatic analyses have not revealed large-scale conservation of the HT signal motif in any sequenced organism to date, with the exception of *Phytophthora* spp. (Bhattacharjee et al., 2006). Even the related apicomplexan *Toxoplasma gondii*, also an obligate intracellular parasite that resides within a PV, encodes very few proteins with the HT signal in the correct N-terminal position. Fusion proteins consisting of the signal sequence of the *T. gondii* protein GRA7, the HT signal from two different *P. falciparum* proteins, and the GFP are not exported into the host cell cytoplasm to any detectable level, but remain in the *T. gondii* PV (C. van Ooij, K. Haldar and L. Knoll, unpublished). If *T. gondii* contains an export apparatus, it has evolved to recognize different signals.

Considering the difference in host cell (nucleated in the case of *Toxoplasma*, enucleated for most *Plasmodium* species) it is possible that the functions performed by the exported *Plasmodium* proteins are already present in a nucleated host cell, or that a nucleated cell is capable of making the necessary alterations to accommodate the growing PV. In addition, any exported *T. gondii* protein is subject to presentation by the host's MHC class I, a process absent in red blood cells.

Only the plant pathogens of the genus *Phytophthora* are known to possess an export system similar to that of *Plasmodium* (Bhattacharjee et al., 2006). These pathogenic oomycetes, which include the causative agent of potato blight, are known to export proteins into the cytoplasm of the host cells. The sequence RxLR is conserved in the putative effector proteins. Protein fusions of the N-terminal region of *Phytophthora* proteins (including RxLR) and GFP are exported from *P. falciparum* into the erythrocyte (Bhattacharjee et al., 2006). Comparing the predicted secretomes of the oomycete and *Plasmodium*, one finds little conservation of effectors. This is most likely a reflection of the differences in the host cells: the erythrocyte and the plant cell. As the two genera are far removed evolutionarily, it remains to be determined if the equivalent signals and export systems (which remain unknown, see below) are derived from a common ancestor or is an example of convergent evolution.

Mechanism of transport

Nature of transporter

Although the signal for export is clear, the mechanism by which proteins are transported from the parasite into the host is not. It has been suggested that the HT signal has the intrinsic property of passing through membranes (Romisch, 2005), similar to antennapedia and the HIV tat protein (Dietz and Bahr, 2004). A problem with this model is that it remains to be explained why the HT signal can only promote passage past the PVM, and not the parasite plasma membrane or the erythrocyte membrane, and how it acts in proteins with multiple transmembrane segments. More likely, a proteinaceous transporter or transporter complex in the PVM is responsible for exporting proteins, similar to the import of proteins into the mitochondria and chloroplasts. No homologues of known organellar transport systems that could be dedicated to protein export have been reported.

Role of heat shock proteins

Several transport systems rely on heat shock proteins or chaperones for either delivery of proteins to the transport apparatus (Type III), or both delivery and extrusion (mitochondria). In the case of the Type III secretion apparatus, effectors are bound by chaperones (some of which are specific for individual effectors) that prevent their folding and deliver them to the transport apparatus. In the case of mitochondrial import in mammals, Hsp70 and
Hsp90 deliver preproteins to the mitochondrial import complex through a specific interaction with TOM70, a part of the import machinery (Young et al., 2003). The ATPase activity of Hsp90 then promotes the translocation of the protein. In the matrix of the mitochondrion, binding of mitochondrial Hsp70 to the protein as it protrudes from the transporter provides the energy for the translocation (Ungermann et al., 1994), which presumably also helps the protein assume its correct conformation. Import of proteins into the chloroplast and the peroxisome similarly rely on chaperones to deliver proteins to the organelle (reviewed in Jackson-Constan et al., 2001; Michels et al., 2005). It is highly likely that export of Plasmodial proteins similarly relies on Hsps. Proteomic analysis by Nyalwidhe and Lingelbach (2006) showed that the largest group of proteins in the PV was the Hsps. We speculate that these play a role in the transit of exported proteins through the PV space by keeping them in a transport-competent state and delivering them to the transporter. As mentioned above, several J-domain-containing proteins, which function as accessory proteins to Hsp70, are exported. Interestingly, erythrocyte Hsp70 undergoes a shift from a soluble to membrane-bound state in infected erythrocytes (Banumathy et al., 2002). It is possible that the parasite J-domain-containing proteins are recruiting host Hsp70 to promote the translocation of parasite effectors.

**Future directions**

The two main remaining questions in *Plasmodium* protein export are the identity of the transporter that moves the proteins across the PVM and the functions of the exported proteins. Answering these questions will provide a much clearer picture of the way the parasite modifies its host cell for intracellular survival and persistence within the host. Given their conservation across parasite species, these proteins may be essential for parasite survival, which together with the lack of similarity to host proteins, makes them, and the transporter itself, excellent drug targets.

**References**


